[a] Values in parentheses correspond to the highest resolution shell

[0513] CD40L formed a trimer (polypeptides A, B, and C in FIG. 17A). Each 309 Tn3 scaffold (polypeptides D, E, and F in FIG. 17A) made contact with two CD40L polypeptides. The crystal structure revealed that there are six specific contacts between each 309 scaffold and the first and second CD40L polypeptides. Aspartic acid 17 in the BC makes contact with threonine 251 in the first CD40L. Glutamic acid 18 in the BC loop makes contact with arginine 203 in the first CD40L and with isoleucine 204 in the second CD40L. Serine 47 in the DE loop makes contact with histidine 249 in the first CD40L. Tryptophan 49 in the DE loop makes contact with valine 247 in the first CD40L. Aspartic acid 70 in the FG loop makes contact with serine 185 in the second CD40L (see FIG. 17A). CD40L amino acid residues contacting the 311 scaffold are also shown in FIG. 18A.

[0514] An in the case of 309, each 311K4E_12 monomer scaffold (polypeptides A, B, and C in FIG. 17B) makes contact with two CD40L polypeptides. The crystal structure revealed that there are 19 specific contacts between each 311K4E_12 scaffold and the first and second CD40L polypeptides. Asparagine 17 in the BC loop makes contacts with tyrosine 146 and glutamic acid 142 in the first CD40L. Arginine 18 in the BC loop makes contact with glutamic acid 142, tyrosine 146, and methionine 148 in the first CD40L. Serine 19 in the BC loop makes contact with glutamic acid 142 and leucine 155 in the first CD40L. Serine 22 in the BC loop makes contact with asparagine 151 in the first CD40L. Histidine 15 in the BC loop makes contact with tyrosine 146 in the first CD40L. Histidine 51 in the DE loop makes contact with tyrosine 146 in the first CD40L and with glutamic acid 230 in the second CD40L. Valine 50 in the DE loop makes contact with glutamic acid 230 in the second CD40L. The N-terminal region of the 311K4E_12 monomer scaffold is connected to the second CD40L. Arginine 200 in the second CD40L makes contact with threonine 7, aspartic acid 8, and threonine 10 in the N-terminal region of 311K4E_12. Arginine 203 in the second CD40L makes contact with glutamic acid 4 and aspartic acid 5. CD40L amino acid residues contacting the 309 scaffold are also shown in FIG. 18B.

[0515] The crystal structures of CD40L in complexes with 309 and 311K4E_12 showed that the 311K4E_12 and 309 monomer scaffolds bind to different epitopes located in different parts of the CD40L trimer complex (FIG. 17C). The structures showed that both scaffolds bind in the same groove that would interact with the CD40 receptor.

[0516] The crystal structure of a 342 with CD40L is provided in Figure 20 and shows that while 342 binds on the same part of CD40L specific changes in the contact

residues are seen as compared to the parental 309 clone. Specifically, in 342 aspartate 18 in the BC loop makes contact with threonine 251 of CD40L and histidine 47 of the DE loop makes contact with histidine 249 of CD40L, histidine 48 of the DE loop makes contact with histidine 249, serine 245 and serine 248 of CD40L, and histidine 50 of the DE loop makes contact with valine 247 of CD40L.

[0517] The crystal structure of a 342 and 311K4E_12 with CD40L demonstrates that both scaffolds can bind simultaneously to their respective epitopes which are located in different parts of the CD40L trimer complex (FIG 21). The contacts for each of the separate scaffolds (as described above) are maintained.

[0518] The examples shown above illustrate various aspects of the invention and practice of the methods of the invention. These examples are not intended to provide an exhaustive description of the many different embodiments of the invention. Thus, although the invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, those of ordinary skill in the art will realize readily that many changes and modifications can be made without departing from the spirit or scope of the appended claims.

SEQUENCES

SEQ ID NO: 1
CD40L sp|P29965|CD40L_HUMAN - Membrane form
Cytoplasmic domain = 1-20
Signal anchor type II membrane protein region = 21-46
Soluble form = 113-261
MIETYNQTSPRSAATGLPISMKIFMYLLTVFLITQMIGSALFAVYLHRRLDKIEDERNLH
EDFVFMKTIQRCNTGERSLSLLNCEEIKSQFEGFVKDIMLNKEETKKENSFEMQKGDQNP
QIAAHVISEASSKTTSVLQWAEKGYYTMSNNLVTLENGKQLTVKRQGLYYIYAQVTFCSN
REASSQAPFIASLCLKSPGRFERILLRAANTHSSAKPCGQQSIHLGGVFELQPGASVFVN
VTDPSQVSHGTGFTSFGLLKL

SEQ ID NO: 2
CD40L- Soluble form, corresponds also to the co-crystallized construct
MQKGDQNPQIAAHVISEASSKTTSVLQWAEKGYYTMSNNLVTLENGKQLTVKRQGLYYIY
AQVTFCSNREASSQAPFIASLCLKSPGRFERILLRAANTHSSAKPCGQQSIHLGGVFELQ
PGASVFVNVTDPSQVSHGTGFTSFGLLKL

SEQ ID No: 3
Tn3 (with unmodified loops)
IEVKDVTDTTALITWFKPLAEIDGCELTYGIKDVPGDRTTIDLTEDENQYSIGNLKPDTE

WHAT IS CLAIMED IS:

1. A Tn3 scaffold comprising a CD40L-specific monomer subunit, wherein the monomer subunit comprises seven beta strands designated A, B, C, D, E, F, and G, and six loop regions designated AB, BC, CD, DE, EF, and FG, and wherein the Tn3 scaffold specifically binds to a CD40L epitope comprising amino acids located at positions 142 to 155, 200 to 230, or 247 to 251 of SEQ ID NO: 2.

2. The Tn3 scaffold of claim 1, comprising a single CD40L-specific monomer subunit.

3. The Tn3 scaffold of claim 1, comprising two CD40L-specific monomer subunits connected in tandem.

4. The Tn3 scaffold of claim 3, wherein the two CD40L-specific monomer subunits are directly connected.

5. The Tn3 scaffold of claim 3, wherein the two CD40L-specific monomer subunits are connected by a linker.

6. The Tn3 scaffold of claim 5, wherein the linker comprises a peptide linker.

7. The Tn3 scaffold of claim 6, wherein the peptide linker is a flexible peptide linker.

8. The Tn3 scaffold of claim 7, wherein the peptide linker comprises a $(G_mX)_n$ sequence wherein:

   (a) X is Serine (S), Alanine (A), Glycine (G), Leu (L), Isoleucine (I), or Valine (V);

   (b) m and n are integers;

   (c) m is 1, 2, 3 or 4; and

   (d) n is 1, 2, 3, 4, 5, 6, or 7.

9. The Tn3 scaffold of claim 8, wherein the peptide linker comprises SEQ ID NO: 131, SEQ ID NO: 132, SEQ ID NO: 142 or SEQ ID NO: 143.

10. The Tn3 scaffold of claim 3, wherein the binding of the scaffold to CD40L is improved over that of a Tn3 scaffold comprising a single CD40L-specific monomer subunit.

11. The Tn3 scaffold of claim 3, wherein the binding of the scaffold to CD40L improves the action on the target over that of a Tn3 scaffold comprising a single CD40L-specific monomer subunit.

12. The Tn3 scaffold of claim 10, wherein the improvement in binding to CD40L is an improvement in binding affinity, an improvement in binding avidity, or both.

13. The Tn3 scaffold of claim 10 wherein binding affinity for CD40L and protein stability are improved over those of a Tn3 scaffold comprising a single CD40L-specific monomer subunit.

14. The Tn3 scaffold of claim 10, wherein binding avidity for CD40L and protein stability are improved over those of a Tn3 scaffold comprising a single CD40L-specific monomer subunit.

15. The Tn3 scaffold of any one of claims 1-9, wherein at least one CD40L-specific monomer subunit is bound to a linker, or to a heterologous moiety.

16. The Tn3 scaffold of claim 15, wherein a linker or a heterologous moiety is conjugated to the N-terminus or the C-terminus of the scaffold.

17. The Tn3 scaffold of claim 15, wherein the linker comprises a functional moiety.

18. The Tn3 scaffold of claim 17, wherein the functional moiety is an immunoglobulin or a fragment thereof.

19. The Tn3 scaffold of claim 18, wherein the immunoglobulin or fragment thereof comprises an Fc domain.

20. The Tn3 scaffold of claim 19, wherein the Fc domain fails to induce at least one FcγR-mediated effector function.

21. The Tn3 scaffold of claim 20, wherein said at least one FcγR-mediated effector function is ADCC.

22. The Tn3 scaffold of claim 15, wherein at least one CD40L-specific monomer subunit is bound to a heterologous moiety.

23. The Tn3 scaffold of any one of claims 3-14, comprising at least two identical CD40L-specific monomer subunits.

24. The Tn3 scaffold of any one of claims 3-14, wherein at least two CD40L-specific monomer subunits specifically bind to different CD40L epitopes.

25. The Tn3 scaffold of claim 24, wherein the different CD40L epitopes are non-overlapping epitopes.

26. The Tn3 scaffold of claim 24, wherein the different CD40L epitopes are overlapping epitopes.

27. The Tn3 scaffold of claim 1, wherein the beta strands of at least one CD40L-specific monomer subunit have at least 90% sequence identity to the beta strands of SEQ ID NO: 3.

28. The Tn3 scaffold of claim 1, wherein the A beta strand comprises SEQ ID NO: 11 except for at least one mutation.

29. The Tn3 scaffold of claim 1, wherein the B beta strand comprises SEQ ID NO: 12 except for at least one mutation.

30. The Tn3 scaffold of claim 1, wherein the C beta strand comprises SEQ ID NO: 13 or 14 except for at least one mutation, and wherein the cysteine in SEQ ID NO: 13 or 14 is not substituted.

31. The Tn3 scaffold of claim 1, wherein the D beta strand comprises SEQ ID NO: 15 except for at least one mutation.

32. The Tn3 scaffold of claim 1, wherein the E beta strand comprises SEQ ID NO: 16 except for at least one mutation.

33. The Tn3 scaffold of claim 1, wherein the F beta strand comprises SEQ ID NO: 17 except for at least one mutation, and wherein the cysteine in SEQ ID NO: 17 is not substituted.

34. The Tn3 scaffold of claim 1, wherein the G beta strand comprises SEQ ID NO: 18 except for at least one mutation.

[a] Values in parentheses correspond to the highest resolution shell

[0513] CD40L formed a trimer (polypeptides A, B, and C in FIG. 17A). Each 309 Tn3 scaffold (polypeptides D, E, and F in FIG. 17A) made contact with two CD40L polypeptides. The crystal structure revealed that there are six specific contacts between each 309 scaffold and the first and second CD40L polypeptides. Aspartic acid 17 in the BC makes contact with threonine 251 in the first CD40L. Glutamic acid 18 in the BC loop makes contact with arginine 203 in the first CD40L and with isoleucine 204 in the second CD40L. Serine 47 in the DE loop makes contact with histidine 249 in the first CD40L. Tryptophan 49 in the DE loop makes contact with valine 247 in the first CD40L. Aspartic acid 70 in the FG loop makes contact with serine 185 in the second CD40L (see FIG. 17A). CD40L amino acid residues contacting the 311 scaffold are also shown in FIG. 18A.

[0514] An in the case of 309, each 311K4E_12 monomer scaffold (polypeptides A, B, and C in FIG. 17B) makes contact with two CD40L polypeptides. The crystal structure revealed that there are 19 specific contacts between each 311K4E_12 scaffold and the first and second CD40L polypeptides. Asparagine 17 in the BC loop makes contacts with tyrosine 146 and glutamic acid 142 in the first CD40L. Arginine 18 in the BC loop makes contact with glutamic acid 142, tyrosine 146, and methionine 148 in the first CD40L. Serine 19 in the BC loop makes contact with glutamic acid 142 and leucine 155 in the first CD40L. Serine 22 in the BC loop makes contact with asparagine 151 in the first CD40L. Histidine 15 in the BC loop makes contact with tyrosine 146 in the first CD40L. Histidine 51 in the DE loop makes contact with tyrosine 146 in the first CD40L and with glutamic acid 230 in the second CD40L. Valine 50 in the DE loop makes contact with glutamic acid 230 in the second CD40L. The N-terminal region of the 311K4E_12 monomer scaffold is connected to the second CD40L. Arginine 200 in the second CD40L makes contact with threonine 7, aspartic acid 8, and threonine 10 in the N-terminal region of 311K4E_12. Arginine 203 in the second CD40L makes contact with glutamic acid 4 and aspartic acid 5. CD40L amino acid residues contacting the 309 scaffold are also shown in FIG. 18B.

[0515] The crystal structures of CD40L in complexes with 309 and 311K4E_12 showed that the 311K4E_12 and 309 monomer scaffolds bind to different epitopes located in different parts of the CD40L trimer complex (FIG. 17C). The structures showed that both scaffolds bind in the same groove that would interact with the CD40 receptor.

[0516] The crystal structure of a 342 with CD40L is provided in Figure 17D and shows that while 342 binds on the same part of CD40L specific changes in the contact

residues are seen as compared to the parental 309 clone. Specifically, in 342 aspartate 18 in the BC loop makes contact with threonine 251 of CD40L and histidine 47 of the DE loop makes contact with histidine 249 of CD40L, histidine 48 of the DE loop makes contact with histidine 249, serine 245 and serine 248 of CD40L, and histidine 50 of the DE loop makes contact with valine 247 of CD40L.

[0517] The crystal structure of a 342 and 311K4E_12 with CD40L demonstrates that both scaffolds can bind simultaneously to their respective epitopes which are located in different parts of the CD40L trimer complex (FIG 17E). The contacts for each of the separate scaffolds (as described above) are maintained.

[0518] The examples shown above illustrate various aspects of the invention and practice of the methods of the invention. These examples are not intended to provide an exhaustive description of the many different embodiments of the invention. Thus, although the invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, those of ordinary skill in the art will realize readily that many changes and modifications can be made without departing from the spirit or scope of the appended claims.

SEQUENCES

SEQ ID NO: 1
CD40L sp|P29965|CD40L_HUMAN - Membrane form
Cytoplasmic domain = 1-20
Signal anchor type II membrane protein region = 21-46
Soluble form = 113-261
MIETYNQTSPRSAATGLPISMKIFMYLLTVFLITQMIGSALFAVYLHRRLDKIEDERNLH
EDFVFMKTIQRCNTGERSLSLLNCEEIKSQFEGFVKDIMLNKEETKKENSFEMQKGDQNP
QIAAHVISEASSKTTSVLQWAEKGYYTMSNNLVTLENGKQLTVKRQGLYYIYAQVTFCSN
REASSQAPFIASLCLKSPGRFERILLRAANTHSSAKPCGQQSIHLGGVFELQPGASVFVN
VTDPSQVSHGTGFTSFGLLKL

SEQ ID NO: 2
CD40L- Soluble form, corresponds also to the co-crystallized construct
MQKGDQNPQIAAHVISEASSKTTSVLQWAEKGYYTMSNNLVTLENGKQLTVKRQGLYYIY
AQVTFCSNREASSQAPFIASLCLKSPGRFERILLRAANTHSSAKPCGQQSIHLGGVFELQ
PGASVFVNVTDPSQVSHGTGFTSFGLLKL

SEQ ID No: 3
Tn3 (with unmodified loops)
IEVKDVTDTTALITWFKPLAEIDGCELTYGIKDVPGDRTTIDLTEDENQYSIGNLKPDTE

WHAT IS CLAIMED IS:

1. A Tn3 scaffold comprising a CD40L-specific monomer subunit, wherein the monomer subunit comprises seven beta strands designated A, B, C, D, E, F, and G, and six loop regions designated AB, BC, CD, DE, EF, and FG, and wherein the Tn3 scaffold specifically binds to a CD40L epitope comprising amino acids located at positions 142 to 155, 200 to 230, or 247 to 251 of SEQ ID NO: 1.

2. The Tn3 scaffold of claim 1, comprising a single CD40L-specific monomer subunit.

3. The Tn3 scaffold of claim 1, comprising two CD40L-specific monomer subunits connected in tandem.

4. The Tn3 scaffold of claim 3, wherein the two CD40L-specific monomer subunits are directly connected.

5. The Tn3 scaffold of claim 3, wherein the two CD40L-specific monomer subunits are connected by a linker.

6. The Tn3 scaffold of claim 5, wherein the linker comprises a peptide linker.

7. The Tn3 scaffold of claim 6, wherein the peptide linker is a flexible peptide linker.

8. The Tn3 scaffold of claim 7, wherein the peptide linker comprises a $(G_mX)_n$ sequence wherein:

   (a) X is Serine (S), Alanine (A), Glycine (G), Leu (L), Isoleucine (I), or Valine (V);

   (b) m and n are integers;

   (c) m is 1, 2, 3 or 4; and

   (d) n is 1, 2, 3, 4, 5, 6, or 7.

9. The Tn3 scaffold of claim 8, wherein the peptide linker comprises SEQ ID NO: 131, SEQ ID NO: 132, SEQ ID NO: 142 or SEQ ID NO: 143.

10. The Tn3 scaffold of claim 3, wherein the binding of the scaffold to CD40L is improved over that of a Tn3 scaffold comprising a single CD40L-specific monomer subunit.

11. The Tn3 scaffold of claim 3, wherein the binding of the scaffold to CD40L improves the action on the target over that of a Tn3 scaffold comprising a single CD40L-specific monomer subunit.

12. The Tn3 scaffold of claim 10, wherein the improvement in binding to CD40L is an improvement in binding affinity, an improvement in binding avidity, or both.

13. The Tn3 scaffold of claim 10 wherein binding affinity for CD40L and protein stability are improved over those of a Tn3 scaffold comprising a single CD40L-specific monomer subunit.

14. The Tn3 scaffold of claim 10, wherein binding avidity for CD40L and protein stability are improved over those of a Tn3 scaffold comprising a single CD40L-specific monomer subunit.

15. The Tn3 scaffold of any one of claims 1-9, wherein at least one CD40L-specific monomer subunit is bound to a linker, or to a heterologous moiety.

16. The Tn3 scaffold of claim 15, wherein a linker or a heterologous moiety is conjugated to the N-terminus or the C-terminus of the scaffold.

17. The Tn3 scaffold of claim 15, wherein the linker comprises a functional moiety.

18. The Tn3 scaffold of claim 17, wherein the functional moiety is an immunoglobulin or a fragment thereof.

19. The Tn3 scaffold of claim 18, wherein the immunoglobulin or fragment thereof comprises an Fc domain.

20. The Tn3 scaffold of claim 19, wherein the Fc domain fails to induce at least one FcγR-mediated effector function.

21. The Tn3 scaffold of claim 20, wherein said at least one FcγR-mediated effector function is ADCC.

22. The Tn3 scaffold of claim 15, wherein at least one CD40L-specific monomer subunit is bound to a heterologous moiety.

23. The Tn3 scaffold of any one of claims 3-14, comprising at least two identical CD40L-specific monomer subunits.

24. The Tn3 scaffold of any one of claims 3-14, wherein at least two CD40L-specific monomer subunits specifically bind to different CD40L epitopes.

25. The Tn3 scaffold of claim 1, wherein the beta strands of at least one CD40L-specific monomer subunit have at least 90% sequence identity to the beta strands of SEQ ID NO: 3.

26. The Tn3 scaffold of claim 1, wherein the A beta strand comprises SEQ ID NO: 11 except for at least one mutation.

27. The Tn3 scaffold of claim 1, wherein the B beta strand comprises SEQ ID NO: 12 except for at least one mutation.

28. The Tn3 scaffold of claim 1, wherein the C beta strand comprises SEQ ID NO: 13 or 14 except for at least one mutation, and wherein the cysteine in SEQ ID NO: 13 or 14 is not substituted.

29. The Tn3 scaffold of claim 1, wherein the D beta strand comprises SEQ ID NO: 15 except for at least one mutation.

30. The Tn3 scaffold of claim 1, wherein the E beta strand comprises SEQ ID NO: 16 except for at least one mutation.

31. The Tn3 scaffold of claim 1, wherein the F beta strand comprises SEQ ID NO: 17 except for at least one mutation, and wherein the cysteine in SEQ ID NO: 17 is not substituted.

32. The Tn3 scaffold of claim 1, wherein the G beta strand comprises SEQ ID NO: 18 except for at least one mutation.